SPECIFICATION

NEW AMINOPEPTIDASE AND THE GENES THEREOF

Background of the Invention

The present invention relates to aminopeptidases and genes encoding them.

Koji molds are used for the preparation of soy sauce, *miso* (fermented soybean paste) and other natural seasonings containing protein hydrolysates. For example, soy sauce is produced in two steps, i. e. a step of preparing *koji* and fermentation step. The starting materials are hydrolyzed by an enzyme produced by a *koji* mold (a filamentous fungus belonging to the genus Aspergillus) in the step of preparing the *koji* mold. For improving the taste of the soy sauce, it is important to increase the amount of free amino acids in soy sauce in these steps.

Amino acids are produced from the starting protein by two steps. The first step is the release of peptides from proteins by proteases and the second step is the production of amino acids through hydrolysis of peptides, catalyzed by peptidases.

As for peptidases of *koji* mold, those derived from *Aspergillus oryzae* and *Aspergillus sojae* were reported (JP-Kokai No. 11-346777, DE 95-1952648, WO 9851163, WO 9628542, WO 9615504, WO 9851803 and WO 9814599). It is described therein that leucine aminopeptidase is particularly important in the preparation of soy sauce. However, it has not been reported that known leucine aminopeptidase is resistant to salt. As for the genes of leucine aminopeptidase of the genus *Aspergillus, Kaifu* et al. reported *Aspergillus sojae* (JP-Kokai No. 11-346777), but there has been no report on the salt-resistance of this enzyme.

As for the genus Bacillus, there is a reported of salt-resistant leucine aminopeptidase (Lee, G. D. et al., J. Appl. Microbiol. (1988), 85 (3)).

On the other hand, *Asano* et al. noted that storage proteins in soybean are hydrolyzed into amino acids in a very short period of time in the course of the germination thereof. They found peptidases (aminopeptidase GX capable of efficiently hydrolyzing acidic amino acid-containing peptides and leucine aminopeptidases) in

soybean cotyledons and succeeded in effectively hydrolyzing soybean protein (JP-Kokai No. Hei 9-294583).

In view of the enzymologic properties of aminopeptidase GX of soybean, aminopeptidase GX was a new aminopeptidase that had never been reported. The presence of the aminopeptidase GX of soybean had not been known except in germinating soybean. Aminopeptidase GX of soybean has an activity of effectively releasing N-terminal acidic amino acids from peptides having acidic amino acids such as glutamic acid at the N-terminal thereof. Accordingly, it is possible to produce soy sauce having a high free glutamic acid content and an excellent taste, taking advantage of the effect of this enzyme.

Ninomiya et al. succeeded in producing a large amount of soybean aminopeptidase GX by a genetic recombination technique (JP-Kokai No. 2000-325090). However, it is difficult to use aminopeptidase GX of soybean produced by this method for the production of soy sauce because of the problems of GMO and costs.

Summary of the Invention

The object of the present invention is to provide an aminopeptidase derived from *koji* molds, which is effective in producing soy sauce or protein hydrolysates having a high free amino acid content and an excellent seasoning property, and also a gene encoding the aminopeptidase.

After intensive investigations made for the purpose of solving the above-described problems, the inventors have succeeded in obtaining DNA encoding a new aminopeptidase from *Aspergillus nidulans* by screening the genome DNA library of *Aspergillus nidulans* with *A. nidulans* EST homologous to soybean aminopeptidase GX genes as the probe. The present invention has been completed on the basis of this fact.

Namely, the present invention provides a protein of any of the following items (A) to (D):

(A) a protein having the amino acid sequence represented by amino acid Nos. 1 to 519 in SEQ ID NO: 2,

(B) a protein having the amino acid sequence represented by amino acid Nos. 1 to 510 in SEQ ID NO: 4,

(C) a protein having the amino acid sequence corresponding to amino acid Nos. 1 to 519 in SEQ ID NO: 2, wherein one or more amino acid(s) are substituted, deleted, inserted, added or inverted in the sequence of SEQ ID NO:2, and which protein has an activity of catalyzing the reaction for releasing an amino acid from the N-terminal of a peptide, or

(D) a protein having an amino acid sequence corresponding to amino acid Nos. 1 to 510 in SEQ ID NO: 4, wherein one or more amino acid(s) are substituted, deleted, inserted, added or inverted in the sequence of SEQ ID:4, and which protein has an activity of catalyzing the reaction for releasing an amino acid from the N-terminal of a peptide,

The present invention also provides nucleic acid molecules encoding any of the aforementioned proteins (A) to (D), recombinant nucleic acid molecules containing these nucleic acid molecules, transformed microorganism hosts and a process for producing an aminopeptidase by using the transformed microorganism hosts. In the present invention, the transformed microorganism hosts include transformed filamentous fungi, in particular, transformed *koji* molds.

The present invention further provides an aminopeptidase having the following properties 1) to 8):

1) The aminopeptidase hydrolyzes a peptide or protein having leucine or methionine at the N-terminal to release leucine or methionine;

2) The aminopeptidase has an optimum pH of about 7.0 to 7.5;

3) The aminopeptidase has an optimum temperature of about 37 to 45°C;

4) The aminopeptidase has a remaining activity of at least 80 % even at a sodium chloride concentration of 3 M, when the activity thereof in the absence of sodium chloride is defined as 100%;

5) The aminopeptidase has a remaining activity of at least 80 % after the storage in the presence of 3 M of sodium chloride at 0°C for 24 hours, when the activity thereof after the storage in the absence of sodium chloride at 0℃ for 24 hours is defined as 100 %;

6) The aminopeptidase has a remaining activity of at least 60 % after the storage at pH

5.8 to 9.5 at 0°C for 24 hours, while the activity thereof after the storage at pH 7.5 at 0°C for 24 hours is defined as 100 %;

7) The aminopeptidase shows a molecular weight of about 550 kD as measured by native PAGE and a molecular weight of 22 or 33 kD as measured by SDS-PAGE after reducing and heating; and,

8) The aminopeptidase requires cobalt ion or zinc ion for the activation.

Brief Description of the Drawings

Fig. 1 is a graph showing the temperature-dependency of PepE activity. The horizontal axis represents the temperature, and the longitudinal axis represents the relative activity of leucine aminopeptidase, while the activity obtained at 37°C is defined as 100.

Fig. 2 is a graph showing the influence of the concentration of sodium chloride in the reaction mixture on PepE. The horizontal axis shows NaCl concentration (M), and the longitudinal axis shows the relative activity of leucine aminopeptidase at varied NaCl concentration, while the activity in the absence of NaCl is defined as 100.

Fig. 3 is a graph showing the dependence of PepE activity on pH. The horizontal axis represents pH, and the longitudinal axis represents the relative activity of leucine aminopeptidase, while the activity in a potassium phosphate buffer (pH 7.5) is 1 defined as 100.

Description of the Preferred Embodiments

As described above, the present invention relates to aminopeptidases from *koji* molds, nucleic acid molecules encoding them, transformed microorganism hosts harboring a recombinant DNA containing the nucleic acid molecules, and a process for producing an aminopeptidase by culturing the transformed microorganism hosts. In the present invention, an aminopeptidase protein from *koji* molds of the invention is represented by "PepE", and the gene encoding PepE is represented by "pepE". The term "aminopeptidase" as used herein indicates a protein having an activity of catalyzing the reaction for successively releasing amino acids from the N-terminal of a

peptide.

The nucleic acid molecules encoding the aminopeptidases of the present invention can be obtained from the chromosomal DNA or cDNA of *Aspergillus nidulans*. Specifically, those nucleic acid molecules can be obtained from the chromosomal DNA library of *Aspergillus nidulans* such as *Aspergillus nidulans* A26. A clone harboring nucleic acid molecules of the present invention can be obtained by PCR (polymerase chain reaction) method using the chromosomal DNA library of *Aspergillus nidulans* as the template by producing PCR primers based on the gene sequence of aminopeptidase GX derived from germinating soybean (JP-Kokai No. 2000-325090) and the nucleotide sequences of EST fragments having a high homology in *Aspergillus nidulans* EST data base. The examples of PCR primers include, for example, oligonucleotides having the nucleotide sequence of SEQ ID NOs: 6 and 7.

The nucleic acid molecules of the present invention can be obtained from a cDNA library produced from poly (A) RNA of *Aspergillus nidulans* by PCR using oligonucleotides having the nucleotide sequence of SEQ ID NOs: 8 and 9 as primers, and by 5'-RACE using oligonucleotides of SEQ ID NOs: 10 and 11 as primers. SEQ ID NO: 1 shows the nucleotide sequence of genomic DNA containing the gene encoding *Aspergillus nidulans* A26 PepE obtained as described above. SEQ ID NO: 2 shows the nucleotide sequence and amino acid sequence of cDNA, and SEQ ID No: 3 shows the amino acid sequence thereof alone. The nucleotide sequences of genomic DNA and cDNA were compared with each other, which revealed that there were no introns in genomic DNA.

The nucleic acid molecules in the present invention may be those capable of encoding the aminopeptidases of the present invention. They include DNA containing the nucleotide sequence of nucleotide position Nos. 72 to 1628 of the nucleotide sequence shown in SEQ ID NO: 2 and also those obtained by removing an unnecessary part locating at the 5' terminal. The term "nucleic acid molecule" includes DNA, RNA and analogues of them. Depending on the purpose for which they are used, those encoding only mature protein are also usable. Nucleic acid molecules of the present invention also include those obtained by replacing a codon encoding an amino

acid in the encoding domain with another equivalent codon. The nucleic acid molecules of the present invention may be those encoding aminopeptidase having the substitution, deletion, insertion, addition or inversion of one or more amino acids at one or plural positions so far as the encoded aminopeptidase activity is not impaired. The meaning of the term "plural", which varies depending on the position and variety of the amino acid residues in the three-dimensional structure of the peptidase protein, herein indicates usually 2 to 300, preferably 2 to 170, more preferably 2 to 50 and most preferably 2 to 10.

The nucleic acid molecule encoding the protein substantially the same as the aminopeptidase described above can be obtained by modifying the nucleotide sequence of pepE such that an amino acid at a particular position is substituted, deleted, inserted or added by, for example, a site-directed mutagenesis. The modified nucleic acid molecules can be obtained by a known process for mutagenesis. The processes for mutagenesis include a process where DNA encoding PepE is treated *in vitro* with hydroxylamine or the like and a process where Escherichia bacteria harboring DNA encoding PepE are irradiated with UV ray or treated with a mutagen usually used for the artificial mutagenesis such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrite.

The above-described substitution, deletion, insertion, addition or inversion of the nucleotide also includes the mutation naturally found in the varieties or strains of the *koji* mold. The nucleic acid molecule having such a mutation is expressed in suitable cells and PepE activity of the expressed product is examined to obtain nucleic acid molecules encoding the protein substantially the same as PepE. Further, nucleic acid molecules encoding the protein substantially the same as PepE protein can be obtained, for example, by isolating a nucleic acid molecules which can hybridize under stringent condition with a nucleic acid molecule having the sequence consisting of nucleotide no. 72 to 1628 of the nucleotide sequence of SEQ ID NO:2 and which encodes a protein having PepE activity. The term "stringent condition" herein indicates the condition under which so-called specific hybrids are formed. It is difficult to clearly numerate the conditions because they vary depending on GC content of each sequence and the presence or absence of repeated sequences. However, the conditions may be, for

example, the condition where nucleic acid molecules having a high homology of, for example, at least 65 %, can hybridize with each other and those having a homology of lower than 65 % do not hybridize with each other. Alternately, the condition may be the one where such nucleic acid molecules hybridize under ordinary washing conditions for the Southern hybridization, i. e. 60℃, 1 x SSC and 0.1 % SDS, preferably 0.1 x SSC and 0.1 % SDS salt concentration. The genes which can hybridize under such conditions may contain those in which a stop codon is formed within the genes or those which lost the activity by a mutation in the active center. They can be easily removed by connecting them with a commercially available activity expression vector and determining PepE activity by a method which will be described below.

The nucleic acid molecules of the present invention can also be obtained from chromosomal DNA or cDNA of microorganisms of another species of the genus *Aspergillus* such as *Aspergillus oryzae.* In particular, they can be obtained from cDNA library of *Aspergillus oryzae* such as *Aspergillus oryzae* RIB40 (ATCC 42149) by PCR method. The nucleic acid molecules can be produced by synthesizing an oligonucleotide primer for PCR based on the nucleotide sequence of PepE of *Aspergillus nidulans* and conducting PCR by using cDNA library prepared from the cells of *Aspergillus oryzae*, e. g. *Aspergillus oryzae* RIB40, as the template. Primers for PCR include the oligonucleotides having the nucleotide sequences of SEQ ID NOs: 12 or 13 for 5'-RACE or the nucleotide sequences of SEQ ID NOs: 14 or 15 for 3'-RACE.

The nucleotide sequence and amino acid sequence of gene cDNA corresponding to pepE of *Aspergillus oryzae* RIB40 obtained as described above are shown in SEQ ID NO: 4 and the amino acid sequence is shown in SEQ ID NO: 5 alone. The amino acid sequence of PepE of *Aspergillus nidulans* shown in SEQ ID NO: 2 and the amino acid sequence of a corresponding aminopeptidase of *Aspergillus oryzae* shown in SEQ ID NO: 4 have a homology of about 77 %, and furthermore, about 120 amino acid residues are different in the mature protein portions. The homology between pepE of *Aspergillus nidulans* and the corresponding gene of *Aspergillus oryzae* was about 71 % for the coding region.

In one embodiment of the present invention, the nucleic acid molecule of the

present invention comprises a nucleic acid molecule encoding a protein having an activity of catalyzing the reaction for releasing an amino acid from a peptide, wherein the protein has the amino acid sequence corresponding to amino acid position Nos. 1 to 510 of SEQ ID NO: 4 which have substitution, deletion, insertion, addition or inversion of one or more amino acids. In another embodiment, the nucleic acid molecules of the present invention include those that hybridize with DNA having a nucleotide sequence of nucleotide position Nos. 73 to 1602 in the nucleotide sequence of SEQ ID NO: 4 under stringent conditions and that encode a protein having an activity of catalyzing the reaction for releasing an amino acid from a peptide.

In Examples given below, the nucleic acid molecules of the present invention are the DNAs obtained as described above. Once the nucleotide sequences of them had been elucidated, it became possible to easily clone nucleic acid molecules encoding corresponding aminopeptidase from the genome DNA of *Aspergillus nidulans* A26, *Aspergillus oryzae* RIB40, or from other strains of *Aspergillus nidulans* or *Aspergillus oryzae* by PCR or hybridization. Accordingly, such nucleic acid molecules are within the scope of the present invention.

The nucleic acid molecules of the present invention are usable for producing the aminopeptidases of the present invention.

The nucleic acid molecules of the present invention are usable for breeding filamentous fungi such as *koji* mold or for producing aminopeptidase PepE. For example, in one embodiment of the present invention, PepE activity can be increased by introducing the DNA encoding aminopeptidase of the present invention into the cells of a filamentous fungus (such as Aspergillus oryzae), preferably as multi-copy DNA. PepE can be produced by expressing the nucleic acid molecules of the present invention in a suitable host. The filamentous fungi such as *koji* molds thus obtained or PepE obtained therefrom are usable for the production of soy sauce, *miso* (fermented soybean paste) and other seasonings containing protein hydrolysates.

The filamentous fungi into which the nucleic acid molecules of the present invention are introduced include the filamentous fungi belonging to the genus *Aspergillus* such as *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus nidulans*;

those of the genus *Neurospora* such as *Neurospora crassa*; and those of the genus *Rhizomucor* such as *Rhizomucor miehei*. The filamentous fungi of the genus *Aspergillus* are particularly preferred.

The vectors for introducing the nucleic acid molecules of the present invention into the above-described filamentous fungi are not particularly limited and those usually used for breeding filamentous fungi are usable. For example, the vectors used for *Aspergillus oryzae* include pUNG (Lee, B. R. et al., Appl. Microbiol. biotechnol., 44, 425-431 (1995)), pMARG (Tsuchiya, K. et al., Appl. Microbiol. Biotechnol., 40, 327-332 (1993)), pUSC (Gomi, K. et al., Agric. Biol. Chem. 51, 2549-2555 (1987)), etc. pUNG has a marker complementing niaD- (defection in nitric acid assimilability) of *Aspergillus oryzae* niaD300 (Minetoki, T. et al., Curr. Genet. 30, 432-438 (1996)); pMARG has a marker complementing argB- (arginine requirement) of *Aspergillus oryzae* M2-3 (Gomi, K. et al., Agric. Biol. Chem., 51(9), 2549-2555 (1987)); and pUSC has a marker complementing sC- (defection in ATP sulfurylase) of *Aspergillus oryzae* NS4 (Yamada, O. et al., Biosci. Biotech. Biochem., 61(8), 1367-1369 (1997)).

Among these vectors, pUNG and pMARG have a promoter for glucoamylase gene (glaA) and α-amylase gene (amyB terminator). By inserting the DNA of the present invention (for example, the region including nucleotide position Nos. 72 to 1628 of SEQ ID NO: 2) into the downstream of the promoter in frame, PepE can be expressed under the control of the promoter. When pUSC is used, since pUSC does not contain a promoter, PepE can be expressed by introducing it into the host filamentous fungus by the co-transformation thereof with a plasmid such as pUC19 containing DNA of the present invention inserted therein.

Vectors, promoters and markers described in literatures shown in Table 1 given below are also usable depending on the host filamentous fungus. In Table 1, the promoters are shown in terms of the enzymes encoded by the genes naturally regulated by the promoters.

Table 1

Literature	Promoter	Marker	Host filamentous fungus
JP-Kokai No. 4-503450	Neutral α-amylase		*Aspergillus niger*
		argB	*Aspergillus niger*
		argB	*Aspergillus nidulans*
		trpC	*Aspergillus nidulans*
		amdS	*Aspergillus nidulans*
		pyr4	*Neurospora crassa*
		DHFR	*Neurospora crassa*
JP-Kokai No. 62-272988	Taka-amylase		*Aspergillus oryzae*
	Aspartic protease		*Rhizomucor miehei*
	Lipase		*Rhizomucor miehei*
	Glucoamylase, lipase		*Aspergillus niger*
	Amylase, glucoamylase, cellulase		
	Protease, glycolytic enzyme		
JP-Kokai No. 7-51067	Taka-amylase		the genus *Aspergillus*
JP-Kokai No. 7-115976	New promoter sequence is given		*Aspergillus oryzae*
JP-Kokai No. 7-59571	New promoter sequence is given		*Aspergillus oryzae*
Nihon NougeiGakkai-shiVol. 71, No. 10 (1997) 1018-1023	α-Amylase (anyB)		*Aspergillus oryzae*
	Glucoamylase (glaA)		*Aspergillus oryzae*
	Glucosidase (agdA)		*Aspergillus oryzae*

For transforming filamentous fungi, any well-known method can be employed in addition to the methods described in the literatures in Table 1. For example, *Aspergillus oryzae* can be transformed as described below.

The cells (conidia) are inoculated in DPY culture medium (2 % of glucose, 1 % of peptone, 0.5 % of yeast extract, pH 5.0), and they are vigorously shaken at 30°C for about 24 hours to conduct the shaking culture. The culture is filtered through Myracloth (CALBIO CHEM Co.) or a sterilized gauze or the like to recover the cells. The cells are washed with sterilized water and thoroughly drained. The cells are placed in a test tube. An enzyme solution [1.0 % Yatalase; Takara Shuzo Co., Ltd.] or 0.5 % NovoZyme (Novo Nordisk) and 0.5 % cellulase (for example, Cellulase Onozuka; Yakult Co., Ltd.), 0.6 M of $(NH_4)_2SO_4$ and 50 mM of malic acid, pH 5.5] are added

thereto and they are gently shaken at 30°C for about 3 hours. The degree of the protoplastization is monitored with a microscope. When good condition is observed, the protoplasts are stored on ice.

The enzymatic reaction mixture is filtered through Myracloth to remove the cell residue. An equal amount of buffer A (1.2 M of sorbitol, 50 mM of $CaCl_2$, 35 mM of NaCl and 10 mM of Tris-HCl, pH 7.5) is added to the protoplast-containing filtrate, and the obtained mixture is placed in ice. After the centrifugation of the mixture at 1,500 to 2,500 rpm at 0°C for 5 to 10 minutes, the centrifugation is slowly stopped. The pellets are washed with buffer A and then suspended in a suitable amount of buffer A. 20 μl or less of DNA solution (5 to 10 μg) is added to 100 to 200 μl of the protoplast suspension, and the obtained suspension is placed in ice for 20 to 30 minutes. 250 μl of buffer B (60 % polyethylene glycol 6000, 50 mM of $CaCl_2$, 10 mM of Tris-HCl, pH 7.5) is added to the obtained mixture. After gentle mixing, additional 250μl of buffer B is added thereto and the obtained mixture is gently mixed. Then 850 μl of buffer B is added to the mixture and they are gently mixed and then left to stand at room temperature for 20 minutes. 10 ml of buffer A is added to the mixture. The test tube is inverted to mix. After the centrifugation at 1,500 to 2,500 rpm at 0°C for 5 to 10 minutes, the pellets are suspended in 500 μl of buffer A.

A suitable amount of the suspension thus obtained is added to 5 ml of the top agar which has been previously aliquoted and pre-warmed and was overlaid on the lower layer medium (selective medium prepared depending on the marker and containing 1.2 M of sorbitol), and cultured at 30°C. The grown cells are subcultured on the selection medium to confirm that they are transformants. It is preferred that recombinant DNA is further prepared from the cells to confirm the introduction of the DNA of the present invention by restriction enzyme analysis or Southern analysis and the like.

The transformants thus obtained are cultured under conditions suitable for the promoter used to express pepE and thereby to obtain PepE. For example, when *Aspergillus oryzae* is used as the host and glucoamylase promoter is used as the promoter, spores of transformed *Aspergillus oryzae* are suspended in a medium

containing wheat bran, potassium phosphate, etc. and they are cultured at about 30°C for about 3 days to produce PepE. If necessary, the culture is diluted with distilled water or the like and then extracted with a homogenizer or the like to obtain a crude enzyme extract containing PepE. The obtained crude extract can be treated by the gel filtration or a chromatography to further purify PepE. PepE thus obtained can be further purified by salting out, isoelectric precipitation, gel filtration, ion chromatography, reversed phase chromatography or the like and used for hydrolyzing proteins. It is also possible to obtain a protein hydrolysate having a high free amino acid content and a strong taste seasoning property by directly mixing a culture product of the transformed microorganism having an improved PepE activity obtained by the introduction of the nucleic acid molecules of the present invention with a proteinous starting material together with a proteolytic enzyme. The proteinous starting materials used herein are, for example, soybean, wheat and wheat gluten. They further include defatted soybean and various processed proteins such as swollen or solubilized proteins and also proteins separated from the various starting materials.

The activity of PepE can be determined by adding 0.02 ml of crude enzyme extract and 0.015 ml of 100 mM zinc chloride to 0.75 ml of 1 mM Leu-pNA (50 mM sodium phosphate buffer, pH 7.5), reacting them at 37°C for 10 minutes, terminating the reaction by the addition of 0.25 ml of 40 % acetic acid and determining the absorbance of the reaction solution at 405 nm. The activities of PepE in various preparations can be compared each other by defining the activity to generate 1 μmol of p-nitroanilide per minute as 1 unit (U) activity.

As for the practical conditions under which the cultured product of the transformed microorganism or crude enzyme is reacted on proteins, for example, a proteinous starting material having a concentration of 0.2 to 50 % is mixed with the cultured product of the transformed microorganism in the presence of a proteolytic enzyme to conduct the reaction at 5 to 60°C for 4 hours to 10 days.

After the completion of the reaction, insoluble matters such as the unreacted proteinous starting material and the cells are removed by an ordinary separation method such as the centrifugation or filtration. If necessary, the product can be

concentrated under reduced pressure or by reverse osmosis or the like, and the concentrated product can be dried or granulated by a drying treatment such as freeze-drying, drying under reduced pressure or spray-drying. Thus, protein hydrolysates having a high free amino acid content and a strong taste seasoning property can be obtained.

Examples

Example 1: Cloning of pepE genome DNA of Aspergillus nidulans

By homology searching using the EST database of *Aspergillus nidulans* (http://www.genome.ou.edu/fungal.html) on the basis of the sequence of aminopeptidase GX derived from germinating soybean, EST obd03a1.f1, which had a high homology, was found.

According to this information, *Aspergillus nidulans* pepE was cloned from *Aspergillus nidulans* genome library as follows.

The *Aspergillus nidulans* genome library was purchased from Fungal Genetics Stock Center (Kansas City, USA). This library had been obtained by cleaving the genomic DNA of *Aspergillus nidulans* with restriction enzymes, ligating the digest to a cosmid vector and introducing it into *Escherichia coli*. The library was screened as follows. *Escherichia coli* clones harboring the intended genes were screened by PCR using the oligonucleotides having the following sequences which had been synthesized according to the nucleotide sequence of EST obd03a1.f1 and *Escherichia coli* containing the cosmid vector as the source of template DNA.

(primer for 5' terminal)

CTC AAA CGG CCA CAT GAC TAC (SEQ ID NO: 6)

(primer for 3' terminal)

GTC T GT TCA AGT GCA TAG CCT G (SEQ ID NO: 7)

<sequence listing free text>

SEQ ID NOs: 6, 7: PCR primer

As for PCR reaction, 25 cycles of the reaction were conducted after the thermal

denaturation at 94°C for 3 minutes. Each cycle of the reaction was conducted at 94°C for 30 seconds, at 52°C for 10 seconds and at 72°C for 30 seconds. As a result, it was elucidated that four clones contained the intended genes. The cosmid vectors were recovered from the clones to determine the nucleotide sequences. The nucleotide sequence and the amino acid sequence encoded by this nucleotide sequence are shown in SEQ ID NO: 2 and the amino acid sequence alone is shown in SEQ ID NO: 3.

Escherichia coli JM109 strain transformed with a plasmid obtained by inserting the gene into plasmid pUC19 was given a private number AJ13856 and deposited in National Institute of Bioscience and Human Technology, National Institute of Advanced Industrial Science and Technology Ministry of Economy, Trade and Industry (presently, National Institute of Advanced Industrial Science and Technology, Chuo No. 6, Higashi 1-1-1, Tsukuba-shi, Ibaraki-ken, Japan, 305-8566) on March 19, 2001 to be preserved with nomination of FERM P-18263. On March 11, 2002, this strain was transferred to the international deposit as FERM BP-7949.

Example 2: Cloning of pepE cDNA from *Aspergillus nidulans*

Aspergillus nidulans A26 was cultured by shaking in 50 ml of YG medium (0.5 % of yeast extract, 2.5 % of glucose, 0.1 % of minor elements*, pH 6.5) at 30℃ for 48 hours (minor elements*: 0.1 % of $FeSO_4 \cdot 7H_2O$, 0.88 % of $ZnSO_4 \cdot 7H_2O$, 0.04 % of $CuSO_4 \cdot 5H_2O$, 0.015 % of $MnSO_4 \cdot 4H_2O$, 0.01 % of $Na_2B_4O_7 \cdot 10H_2O$, 0.005 % of $(NH_4)_6MoO_{24} \cdot 4H_2O$).

The cells were recovered, frozen in liquid nitrogen and crushed in a mortar. The total RNA was prepared from the crushed cells using RNeasy Plant Mini Kit (QIAGEN), and mRNA was prepared using Micro FAST Track Kit (Invitrogen). cDNA was synthesized from mRNA using cDNA Synthesis Kit (Promega), and the cDNA library was prepared with cDNA PCR Library Kit (TaKaRa).

By using the cDNA library as the template, pepE cDNA was cloned by PCR and 5'-RACE using the oligonucleotides, as the primers, which had the following sequences designed based on *Aspergillus nidulans* genomic DNA sequence.

(Primer for 5' terminal)

CAC CAC CAT GAG TCT AAC TTG G (SEQ ID NO: 8)

(Primer for 3' terminal)

GTC TGT TCA AGT GCA TAG CCT G (SEQ ID NO: 9)

(Primer for 5' terminal for 5'-RACE)

CGT GGT ACC ATG GTC TAG AGT (SEQ ID NO: 10)

(Primer for 3' terminal for 5'-RACE)

AAT CGC AGT AAG CCT GCG AG (SEQ ID NO: 11)

<Sequence listing free text>

SEQ ID NOs: 8-11: PCR primer

As for PCR reaction conditions, 30 cycles of the reaction were conducted after the thermal denaturation at 94°C for 9 minutes. Each cycle of the reaction was conducted at 94°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 30 seconds, and finally the reaction was incubated at 72°C for 5 minutes As a result, DNA fragments of about 1800 bp were obtained by PCR with primers of SEQ ID NOs: 8 and 9 and amplification fragments of about 250 bp were obtained by 5'-PACE with primers of SEQ ID NOs: 10 and 11. The nucleotide sequences of those DNA fragments and the amino acid sequences deduced from the nucleotide sequences are shown in SEQ ID NO: 2.

Escherichia coli JM109 strain transformed with the plasmid obtained by inserting the cDNA fragments of the above-described *Aspergillus nidulans* pepE into pBluescript was given a private number AJ13857 and deposited in National Institute of Bioscience and Human Technology, National Institute of Advanced Industrial Science and Technology Ministry of Economy, Trade and Industry (presently, National Institute of Advanced Industrial Science and Technology, Chuo No. 6, Higashi 1-1-1, Tsukuba-shi, Ibaraki-ken, Japan, 305-8566) as FERM P-18264 on March 19, 2001 and was transferred to the International deposition On March 11, 2002, as FERM BP-7950.

Example 3: Cloning of cDNA homologous to *Aspergillus oryzae* pepE

(1) Construction of *Aspergillus oryzae* cDNA library

Aspergillus oryzae RIB40 (ATCC 42149) was cultured in 50 ml of DPY medium at 30°C for 64 hours. The cells were harvested by filtration to recover 1 g thereof. The cells were immediately frozen in liquid nitrogen and crushed in a mortar. The total RNA was prepared from the crushed cells with RNeasy Plant Mini Kit (QIAGEN). Purified mRNA was obtained from the RNA with mRNA Purification Kit (Pharmacia), and the cDNA library was constructed using cDNA PCR Library Kit (TaKaRa) or 3'-RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL).

(2) Screening of *Aspergillus oryzae* cDNA library

The cloning of the cDNA homologous to *Aspergillus oryzae* pepE was conducted by 5'-RACE where oligonucleotides shown in SEQ ID NOs: 12 and 13 were used as the primers and also by 3'-RACE where those shown in SEQ ID NOs: 14 and 15 were used, considering the PepE sequence of *Aspergillus nidulans* obtained in Example 2.

(Primer for 5' terminal for 5'-RACE)

CGT GGT ACC ATG GTC TAG AGT (SEQ ID NO: 12)

(Primer for 3' terminal for 5'-RACE)

CAT GGG CCC AAT GGT TCC GC (SEQ ID NO: 13)

(Primer for 5' terminal for 3'-RACE)

CCA GAT TCG TAA TGA CTC CCG (SEQ ID NO: 14)

(Primer for 3' terminal for 3'-RACE)

CTA CTA CTA CTA GGC CAC GCG TCG ACT AGT AC (SEQ ID NO: 15)

<Sequence listing free text>

SEQ ID NOs: 12-15: PCR primer

As for the PCR reaction of 5'-RACE, 35 cycles of the reaction was conducted after the thermal denaturation at 95°C for 9 minutes. Each cycle of the reaction was conducted at 94°C for 30 seconds, at 53°C for 30 seconds and at 72°C for 1 minute. As a result, *Aspergillus oryzae* pepE fragments of about 1400 bp were obtained. As for the PCR reaction of 3'-RACE, 35 cycles of the reaction was conducted after the thermal denaturation at 95°C for 9 minutes. Each cycle of the reaction was conducted at 94°C for 30 seconds, at 60°C for 30 seconds and at 72°C for 1 minute. As a result, the gene fragments of about 300 b analogous to *Aspergillus oryzae* pepE were obtained.

The nucleotide sequence of the gene fragments was determined to find that they contained the full length sequence homologous to that of pepE. The nucleotide sequence and the amino acid sequence encoded by this nucleotide sequence are shown in SEQ ID NO: 4 and the amino acid sequence alone is shown in ID NO: 5.

Escherichia coli DH5α strain transformed with a plasmid obtained by inserting the gene sequence into plasmid pBluescript was given a private number AJ13858 and deposited in National Institute of Bioscience and Human Technology, National Institute of Advanced Industrial Science and Technology Ministry of Economy (presently, Advanced Industrial Science and Technology, Chuo No. 6, Higashi 1-1-1, Tsukuba-shi, Ibaraki-ken, Japan, 305-8566) on March 19, 2001 as FERM P-18265. On March 11, 2002, this strain was transferred to the international deposit as FERM BP-7951.

Example 4: Expression of pepE in *Aspergillus oryzae*

(1) Preparation of transformed *Aspergillus oryzae*

The pepE cDNA of *Aspergillus oryzae* obtained in Example 3 was ligated to pBluescript at the SmaI site to prepare plasmid pBSAopepE. pepE cDNA was excised from the plasmid with EcoRI and XbaI and then connected to the downstream of the glucoamylase promoter in pUNG1 vector containing niaD marker gene (Lee, B. R. et al., Applied Microbiology Biotechnology, 44, 425-431 (1995)) to obtain the plasmid pNGAPE, which was used for the transformation. The transformation was conducted with 10 μg

of the plasmid DNA.

Conidia of *Aspergillus oryzae* niaD300 strain were inoculated in DPY culture medium. After shaking the culture at 30°C for 24 hours, the culture mixture was filtered through sterilized gauze to recover the cells, which were then washed with sterilized water. The cells were placed in a test tube. 20 ml of an enzyme solution [1.0 % Yatalase; (Takara Shuzo Co., Ltd.)] was added thereto and they are gently shaken at 30°C for 3 hours. The degree of protoplastization was monitored under a microscope and then the protoplasts were stored in ice.

The enzymatic reaction mixture was filtered through Myracloth to remove the cell residue. An equal amount of buffer A (1.2 M of sorbitol, 50 mM of $CaCl_2$, 35 mM of NaCl and 10 mM of Tris-HCl, pH 7.5) was added to the protoplast-containing filtrate, and the obtained mixture was placed in ice. After the centrifugation of the mixture at 1,500 rpm at 0℃ for 5 minutes, the centrifugation was slowly stopped. The pellets were washed with 10 ml of buffer A twice and then suspended in 1 ml of buffer A.

10 μl of DNA solution (10 μg) was added to 100 μl of the protoplast suspension, and the obtained mixture was placed on ice for 30 minutes. 250 μl of buffer B (60 % PEG (polyethylene glycol) 6000, 50 mM of $CaCl_2$, 10 mM of Tris-HCl, pH 7.5) was added to the obtained mixture. After gently mixing, additional 250 μl of buffer B was added thereto and the obtained mixture was gently mixed. Then 850 μl of buffer B was added to the mixture and they are gently mixed and left to stand at room temperature for 20 minutes. Then, 10 ml of buffer A was added to the mixture. The test tube was inverted. After the centrifugation at 1,500 rpm at 0℃ for 5 minutes, the pellets were suspended in 500 μl of buffer A.

The suspension thus obtained was added to 5 ml of top agar medium which had been previously aliquoted and pre-warm and overlaid on Czapek Dox medium (1.2 M of sorbitol, 0.3 % of sodium nitrate, 0.2 % of potassium chloride, 0.1 % of potassium phosphate, 0.05 % of magnesium sulfate heptahydrate, 0.002 % of ferrous sulfate heptahydrate and 2 % of glucose, pH 5.5), and they were cultured at 30°C. Ten strains of the grown cells were inoculated on Czapek Dox medium to obtain the stable transformants.

(2) Production of pepE

The transformant obtained as described above was cultured with wheat bran, and the aminopeptidase activity of the extract obtained therefrom was determined.

20 g of wheat bran, 0.3 g of potassium phosphate and 14 ml of distilled water were thoroughly stirred together. The obtained mixture was placed in an Erlenmeyer flask and autoclaved at 120°C for 30 minutes to produce a medium. 8 ml of sterilized water was poured into a petri dish, in which a plenty of the spores were formed, and they were stirred together to prepare a spore suspension. The spore suspension was sprayed on the medium. The medium on which the spores were inoculated was thoroughly stirred. After culturing at 30°C for 3 days, 10 parts of distilled water was added to 1 part of the wheat bran prepared as described above and they were extracted with a homogenizer for 5 minutes to obtain a crude enzyme extract.

The aminopeptidase activity of the crude enzyme extract prepared as described above was determined as follows: 0.02 ml of the crude enzyme extract and 0.015 ml of 100 mM zinc chloride were added to 0.75 ml of 1 mM Leu-pNA (50 mM sodium phosphate buffer, pH 7.5) to conduct the reaction at 37°C for 10 minutes. 0.25 ml of 40 % acetic acid was added to the reaction mixture to terminate the reaction. The absorbance of the reaction mixture at 405 nm was measured to determine the activity. 1 unit (U) of the enzymatic activity was defined as the activity forming 1 μmol of p-nitroanilide per minute. As a control, a crude enzyme extract was prepared from a transformant obtained by the transformation with vector DNA containing only the marker gene, and the aminopeptidase activity thereof was determined by the same method as that described above.

As a result, a remarkable increase in the aminopeptidase activity was recognized with the strain in which the gene of the present invention was introduced (Table 2). It was thus confirmed that the introduced aminopeptidase gene was actually expressed and that the aminopeptidase was produced.

Table 2 Aminopeptidase activity in crude enzyme extract

	Aminopeptidase activity (per mg of protein)
Transformant strain 1	0.09 U
Transformant strain 2	0.10 U
Transformant strain 3	0.12 U
Transformant strain 4	0.09 U
Transformant strain 5	0.11 U
Control strain 1	0.03 U
Control strain 2	0.02 U
Control strain 3	0.03 U

Example 5: Characterization of PepE

(1) Purification of pepE

20 g of wheat bran medium was placed in a 300 ml flask and then autoclaved at 120°C. for 20 minutes to prepare a medium.

The spore suspension was prepared for the transformant highly expressing PepE prepared in Example 4,. The suspension was inoculated in the medium, thoroughly mixed and cultured . at 30°C for 5 days. During the culture, 48 hours after the start of the culture the medium was cared by stirring.

1 part (w/w) of the wheat bran thus obtained was immersed in a mixture of 10 parts (w/w) of 20 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA and 1 mM PMSF (phenyl methane sulfonyl fluoride). After leaving the obtained mixture to stand at 4°C for 16 hours, the reaction mixture was filtered through a gauze and then centrifuged (7,500 rpm, 4°C., 10 minutes) to obtain a supernatant liquid to be used as a crude enzyme extract.

Ammonium sulfate was added to the crude enzyme extract to obtain 40 % - 60 % ammonium sulfate precipitation fraction. The precipitate was dissolved in 20 mM potassium phosphate buffer (pH 7.4). The obtained solution was filtered through a filter having a pore diameter of 0.45 μm. The filtrate was applied to a desalting column [HiTrap Desalting of Amersham Pharmacia (25 ml)] previously equilibrated with 20 mM potassium phosphate buffer (pH 7.4) and 150 mM NaCl and eluted with the same buffer. The obtained active fraction was concentrated by ultrafiltration.

The sample thus obtained was adsorbed on an anion exchange column [HiTrap Q-sepharose HP of Amersham Pharmacia (25 ml)] previously equilibrated with 20 mM potassium phosphate buffer (pH 7.4), and then washed with the same buffer in an amount of 3 volumes of the column volume. After the completion of the washing, the active fraction was eluted by linearly increasing NaCl concentration of the buffer from 0 M to 1 M in 20 volumes of the column volume to conduct the elution. The active fraction recovered in the effluent was concentrated by ultrafiltration.

The obtained sample was fractionated by the gel filtration chromatography with HiLoad 26/60 Superdex 200 pg (Amersham Pharmacia). The sample was applied to the column previously equilibrated with 20 mM potassium phosphate buffer (pH 7.4) and 150 mM NaCl and the active fraction was recovered by the elution with the same buffer. Thus, purified PepE was obtained after these procedures.

(2) Determination of PepE activity

The aminopeptidase activity of the enzyme extract was determined as follows. 0.02 ml of the crude enzyme extract was added to 0.73 ml of a substrate solution (1 mM Leu-pNA, 50 mM sodium phosphate buffer (pH 7.5), 2 mM cobalt chloride). After conducting the reaction at 37°C for 10 minutes, 0.25 ml of 40 % acetic acid was added to the reaction mixture to terminate the reaction. The absorbance of the reaction solution at 405 nm was determined and the activity was calculated therefrom. An enzymatic activity for forming 1 μmol of p-nitroanilide per minute was defined as 1 unit.

The enzymatic properties of this enzyme are as follows.

(i) Substrate specificity

The hydrolyzing activities for various X-pNA were determined by the above-described method for determining the activity except that Leu-pNA was replaced with X-pNA. The obtained relative activities, on the basis of the activity for Leu-pNA as 100, are shown in Table 3 given below. It was elucidated that this enzyme efficiently hydrolyzed the peptides having Leu at the N-terminal thereof.

Table 3 Substrate specificity of PepE

X-pNA	X											
	Leu	Lys	Met	Asp	Phe	Arg	Val	Ile	Pro	Gly	Glu	Ala
Relative value	100	0	26	0	2	0	1	1	0	1	0	1

(ii) Optimum temperature

LAP activity was determined at a various temperature by the above-described method for determining the activity. The obtained relative activities, on the basis of the activity at 37°C as 100, are shown in Fig. 1.

(iii) Effects of sodium chloride concentration in the reaction solution

LAP activity obtained by adding varied concentration of NaCl was determined by the above-described method for determining the activity. The relative activities, on the basis of the activity in the absence of NaCl as 100, are shown in Fig. 2. It was apparent that the enzyme was capable of retaining its activity even in the presence of a high concentration of sodium chloride.

(iv) Optimum pH

The above-described activity determination method was repeated except that 50 mM sodium phosphate buffer (pH 7.5) was replaced with another pH buffer so that the final concentration thereof would be 50 mM. The LAP activity in potassium phosphate buffer (pH 7.5) was defined to be 100. The activity at each pH is shown in Fig. 3.

(v) pH stability

The purified enzyme was kept in 50 mM buffers having various pH at 0°C for 24 hours and then LAP activity was determined by the above-described activity determination method (pH 7.5). The relative activities, determined on the basis of the activity (100) after the storage in the phosphate buffer of pH 7.5, are shown in Table 4.

Table 4: pH Stability of PepE

Buffer	pH	Relative activity (%)
Sodium acetate	4.7	18
Sodium acetate	5.8	157
Potassium phosphate	6.4	145
Potassium phosphate	7.0	100
Potassium phosphate	7.5	100
Tris	8.0	88
Sodium carbonate	8.8	82
Sodium carbonate	9.5	64

(vi) Stability in sodium chloride solution

The purified enzyme was kept in 0-4 M NaCl, 20 mM phosphate buffer (pH 7.5) at 0°C for 24 hours and then the activity thereof in the reaction mixture having a salt concentration equal to the storage salt concentration was determined. The results are shown in Table 5.

Table 5 Stability of PepE in sodium chloride solution

Salt concentration (M)	Relative activity (%)
0	100
1	85
2	107
3	84

(vii) Effects of metal ion

The hydrolyzing activity for various X-pNA was determined by the above-descried activity determination method except that cobalt chloride was replaced with various divalent metal salts. The relative activity is shown in Table 6 in which the hydrolyzing activity of Leu-pNA in the presence of cobalt chloride is defined as 100.

Table 6 Effects of metal ion on PepE activity

XCl_2	Relative activity (%)
$X = Co^{++}$	100
$X = Ni^{++}$	1
$X = Mn^{++}$	1
$X = Mg^{++}$	1
$X = Cu^{++}$	1
$X = Ca^{++}$	1
$X = Zn^{++}$	11
No additive	2

The present invention provides a means for obtaining a protein hydrolysate having a high free amino acid content and a strong seasoning property. In particular, the present invention provides an aminopeptidase capable of efficiently hydrolyzing peptides in the production of soy sauce having a high sodium chloride content by the fermentation and also a nucleic acid encoding the same. By the present invention, the seasoning property of soy sauce or protein hydrolysates can be further improved.

The hosts containing the nucleic acid molecule of the present invention in a form, which allows the expression of the nucleic acid molecule, are usable for producing the protein of the present invention.